

Infiltration of lymphocytes in the limbic brain following stimulation of subclinical cellular immunity and low dosages of lithium and a cholinergic agent

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Abstract

This experiment was designed to investigate the hypothesis that single small dosages of lithium (1.5 mEq/kg), the muscarinic agent pilocarpine (15 mg/kg) and spinal cord emulsion encourage perivascular infiltration of lymphocytes into the brain even when overt symptoms of experimental allergic encephalomyelitis are not apparent. The brains of rats that had received this small dosage of lithium and pilocarpine exhibited discernable infiltrations of lymphocytes within limbic tracts but no discernable neuronal loss. Although the brains of the rats that displayed overt seizures following larger dosages of lithium (3 mEq/kg) and pilocarpine (30 mg/kg) exhibited the usual pattern of neuronal loss within multiple thalamic and limbic structures and conspicuous foci of lymphocytic infiltration (particularly within the hippocampal formation) the correlation between the numbers of foci and the proportions of neuronal damage in these structures was not significant statistically. These results indicate that infiltrations of lymphocytes into brain parenchyma are not simple artifacts of the amount of neuronal damage and may be sensitive toxicological markers for subclinical interactions between drugs and immune responses. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Both theory (Poser, 1987) and evidence (Aihara et al., 1995) indicate that subtle changes in the blood brain barrier (Matthews, 1991; Kwon and Prineas, 1994) within very specific structures could

be revealed by an abnormal infiltration of mononuclear cells (lymphocytes) into brain parenchyma. Our clinical interest has focused upon the potential toxicological bases of myalgic encephalomyelitis (Polman et al., 1986; Poser, 1992; Goldstein, 1993; Cook and Persinger, 1998) and its relationship to mild to moderate traumatic brain injuries (Roberts et al., 1992; Sweeney, 1992). We have been evaluating Lewis rats that

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had been inoculated with the spinal cord preparation but did not develop any overt behavioral (clinical) signs of experimental allergic encephalomyelitis (EAE). It is considered a proximal model to multiple sclerosis (Alvord et al., 1984).

Because many of the clinical and neuropsychological complications that follow mild to moderate closed-head injuries strongly resemble those associated with electrical anomalies within the limbic system (Roberts et al., 1992; Persinger, 1995), we selected the lithium/pilocarpine model of epilepsy (Honchar et al., 1983) as the trigger to alter the blood–brain-barrier (BBB). The seizures evoked by a single subcutaneous injection of lithium (3 mEq/kg) and pilocarpine (30 m/kg) a few hours later result in both immediate and delayed necrosis of neurons within dozens of diencephalic and telencephalic structures of the rat brain (Persinger et al., 1988; 1993). One-half the concentration of lithium (1.5 mEq/kg) and pilocarpine (15 mg/kg) evoke neither overt seizures nor discernable neuronal damage. In the present experiment we injected concentrations of spinal cord (in complete Freund's adjuvant), not sufficient to evoke overt signs of EAE, and concentrations of lithium/pilocarpine sufficient to either produce or not produce overt seizures and brain damage. We predicted that infiltration of lymphocytes would also be evident within discrete limbic regions of rats that received even the smallest dosages of lithium and this cholinergic agent.

2. Methods and materials

A total of 33 Lewis rats (15 male, 18 female), 120 days of age, were acclimatized to 12:12 h light:dark cycle (onset 0800) within a temperature controlled room. After the approval of the protocol by the university's Animal Care Committee, a total of 8 mg of freshly extracted Lewis male rat spinal cord within 0.2 cc of Freund's complete adjuvant (extracted from 10 cc homogenates of 40 mg of fresh spinal cord/cc) was injected into the hind footpads (0.1 cc per foot) of each rat. Only three rats developed the overt signs of EAE, as

defined by a 5-point severity scale (Missaghi et al., 1992) and were excluded from the study. Consequently there were 30 rats in this study.

Either immediately after the inoculation, 5 days after the inoculation, or, 10 days after the inoculation, a total of 9 rats were injected subcutaneously with 1.5 mEq/kg (1.5 mEq/cc of distilled water) of lithium chloride (Sigma) in the hind flank followed 4 h by a subcutaneous injection into the other flank with 15 mg/kg (15 mg/cc in isotonic saline) of pilocarpine HCl (Sigma). Another nine rats were injected subcutaneously with 3 mEq/kg of lithium chloride (3 mEq/cc of distilled water) into the hind flank followed 4 h later by a subcutaneous injection of 30 mg/kg of pilocarpine (30 mg/cc in isotonic saline) either immediately after inoculation, 5 days later or 10 days later. As a result a total of 18 rats were injected with the combinations of lithium and pilocarpine.

The presence or absence of overt seizures was directly observed. The typical sequence of automatism, ear twitches, head nods, rearing, forelimb clonus and falling (Bureau et al., 1994) occurred within 1 h for all of the rats that received the 3 mEq/kg of lithium and 30 mg/kg of pilocarpine. The rats that received the subclinical dosage (1.5 mEq/kg; 15 mg/kg) did not display any discriminable overt seizure behaviors. Because rats that display overt seizures following the injection of 3 mEq/kg of lithium and 30 mg/kg of pilocarpine usually die within 3 days and acepromazine (Atravet, Ayerst Laboratories) reduces this risk to less than 10%, all of the rats were injected subcutaneously in the intrascapular region with 25 mg/kg of this neuroleptic 60 min after the injection of the pilocarpine.

Four additional rats (two male; two female) that had been inoculated with spinal cord, had not been injected with either lithium or pilocarpine, but had been injected with isotonic saline and acepromazine were used as procedural controls while an additional two naive (normal) rats, randomly selected from the Lewis colony, served as reference controls.

Another six rats were injected intracerebrally with either 1 mg/cc of C 48/80 (a mast cell degranulator) or isotonic saline (Persinger, 1983). This procedure results in massive infiltration of

mononuclear cells (lymphocytes) into the parenchyma around the injection site within the cerebrum only if the rats had been inoculated with the emulsion of spinal cord and Freund's adjuvant 10–15 days previously. Direct comparison of these lymphocytes, at 1000 X magnification, was employed to verify if the cells that were observed in the parenchyma of the rats treated with lithium and pilocarpine, whether seized or not, were morphologically indistinguishable.

After anesthesia with 40 mg/kg of sodium pentobarbital, three rats were injected with 20 μ l of isotonic saline and three rats were injected with 20 μ l of C 48/80 into the region of the right dorsal hippocampal formation. The volumes were injected directly through the skin and skull with a 100 μ l Hamilton syringe. The rats were then inoculated with the spinal cord preparation. During the 15 days after the inoculation all rats were maintained in shoebox cages that contained soft wood shavings. Food and water were available ad libitum.

On day 15 after the inoculation all of the rats were decapitated. The brains were removed within 4 min, fixed in ethanol–formalin acetic acid, processed and embedded in paraffin. Ten micrometer coronal sections were obtained by microtome for every 200 μ m between the posterior commissure and the anterior commissure. The slides were stained with toluidine blue O. This particular fixative, processing and staining procedure delivers consistent and very clear details of the morphology of neurons and the three classes of glial nuclei that are not apparent with 10% formalin fixation and other stains (Persinger, 1979). Lymphocytes and glial nuclei are also clearly distinguishable.

Each structure, as defined by Paxinos and Watson (1986), was examined at 100 X and 400 X magnification. The numbers of foci that contained infiltration of lymphocytes were counted within the left and right component for each structure. The size of the foci was rated along a 1–8 scale; a score of 8 would involve complete saturation of the grid-field (i.e. width of 120 μ m) at 200 X by perivascular infiltrations. The interrater reliability, completed under blind conditions, for the numbers of foci for 20 separate, randomly selected

slides was 0.93 (rho). To control for the variable cross-sectional areas of structures, the numbers of foci per structure were divided by the cross sectional area of each structure as measured by planimeter and verified by values from Paxinos (1985). The proportion of neuronal dropout, according to a rating scale from 0 to 10 (Bureau et al., 1994) was obtained for each structure. The intrinsic validity of this rating scale has been shown in other studies (Persinger et al., 1993, 1994) by the strong ($r > 0.90$) correlation between the proportion of neuronal loss and quantitative measures of behavioral deficits.

The primary design was a two way analysis of variance. The two levels were: dosages of lithium and pilocarpine (sufficient to produce overt seizures or not sufficient to produce overt seizures) and when (same day, 5 days later or 10 days later) the drugs were injected after the inoculation. Because of the range in numbers of infiltration foci, log (base 10) transformations were completed. To discern if there were any large associations between the severity and extent of the damage and the numbers of foci, Spearman rho correlations were completed between the rankings of the proportions of neuronal dropout and numbers of foci per mm² for all 382 structures (191 left side, 191 right side) examined and, separately, for only those structures that contained at least one foci. Spearman rho correlations were also completed between the average width of the foci and the numbers of foci within the structures. Because of the sample size, the P value was set at < 0.01 level to reject the null hypothesis. All analysis involved SPSS software.

3. Results

Two-way analysis of variance demonstrated that the rats in which overt seizures had been induced experimentally by the higher dosage of lithium/pilocarpine displayed significantly more [$F_{1,12} = 92.11$, $P < 0.001$; $\eta^2 = 0.89$] foci of infiltrations of lymphocytes into the parenchyma (total for the ten slides) relative to the rats that had received the diluted dosages of lithium and pilocarpine and had not displayed overt seizures.

The mean and standard deviation for the log (base 10) of the number of foci for the nine rats that displayed the overt seizures were 2.0 and 0.4, respectively, while these values for the nine rats that did not display overt seizures were 0.3 and 0.5.

There was no significant difference [$F_{2,12} = 0.06$, $P > 0.05$] in the numbers of foci as a function of when the seizures were precipitated (same day, 5 days or 10 days after inoculation). The interaction between dosage and delay between the inoculation and the induction of the seizures was not statistically significant. [$F_{2,12} = 5.56$, $0.01 < P < 0.05$]. Additional analysis showed no statistically significant sex effects or two way interactions between sex and the other two treatment conditions.

Table 1 contains a list of the 12 structures, as defined by Paxinos and Watson (1986), that showed the highest density of foci (numbers/mm²) of lymphocytes within the parenchyma. Both the numbers of rats that showed infiltrations and the range of density of the foci are indicated. Fig. 1 shows the typical area around a blood vessel within the piriform cortex of a rat that had been inoculated and injected with isotonic saline compared to a rat that had been inoculated and in which limbic seizures had been induced. The infiltration of lymphocytes around blood vessels

was qualitatively conspicuous in the latter brain.

Although rats that had received the lower dosages of lithium (1.5 mEq/kg) and pilocarpine (15 mg/kg) did not show overt seizures and never showed any discriminable evidence (at 400 X) of neuronal necrosis or gliosis, there were discernable patches of infiltrations of lymphocytes. Infiltrations were specifically found within at least three of the nine rats within the fornix, fimbria and lateral olfactory tract and within at least two rats within the septohypothalamic transition, suprachiasmatic nucleus, lateral septum, basal cerebral peduncle, and medial habenula.

Fig. 2 shows microphotographs of the fornix of: (1) a rat that had been inoculated but injected with isotonic saline and (2) a rat that was inoculated and received the low dosages of lithium and pilocarpine (i.e. no overt limbic seizures). The infiltrations of lymphocytes into the surrounding white matter was obvious. The normal containment of erythrocytes and no perivascular infiltration from the brain of rat that served as a procedural control is shown in Fig. 3B while the perivascular disruption and cell aggregates around blood vessels within the fornix of a rat that received the subclinical dosages of lithium/pilocarpine are shown in Fig. 3A.

The Spearman rho correlation ($\rho = 0.09$) between the numbers of foci and the proportion of

Table 1

The 12 structures that contained the most numerous foci of infiltrations of lymphocytes in rats that displayed overt seizures^a

Structure	Numbers (of nine)	Range of density (mm ²)	Median
Subiculum	6	2.2–10.0	6.7
Dorsal hippocampal fornix	6	0.3–10.4	4.5
Corticoamygdaloid nucleus (posteromedial part)	6	0.7–3.5	2.1
Piriform cortex	6	0.1–3.8	1.0
Dentate gyrus (polymorph)	6	0.4–8.2	0.8
Entorhinal cortex	5	0.8–9.8	1.1
Lateral olfactory tract	5	0.9–3.6	0.9
Fimbria	3	0.3–2.0	0.4
Alveus of hippocampus	3	1.5–2.6	1.7
External capsule	3	0.9–3.0	0.8
Lateral dorsal thalamus (ventrolateral part)	3	0.5–1.1	1.1
Parietal cortex	3	0.2–3.1	2.6

^a The numbers of rats (of nine) that showed infiltrations and their range and median value for density within those areas are indicated.

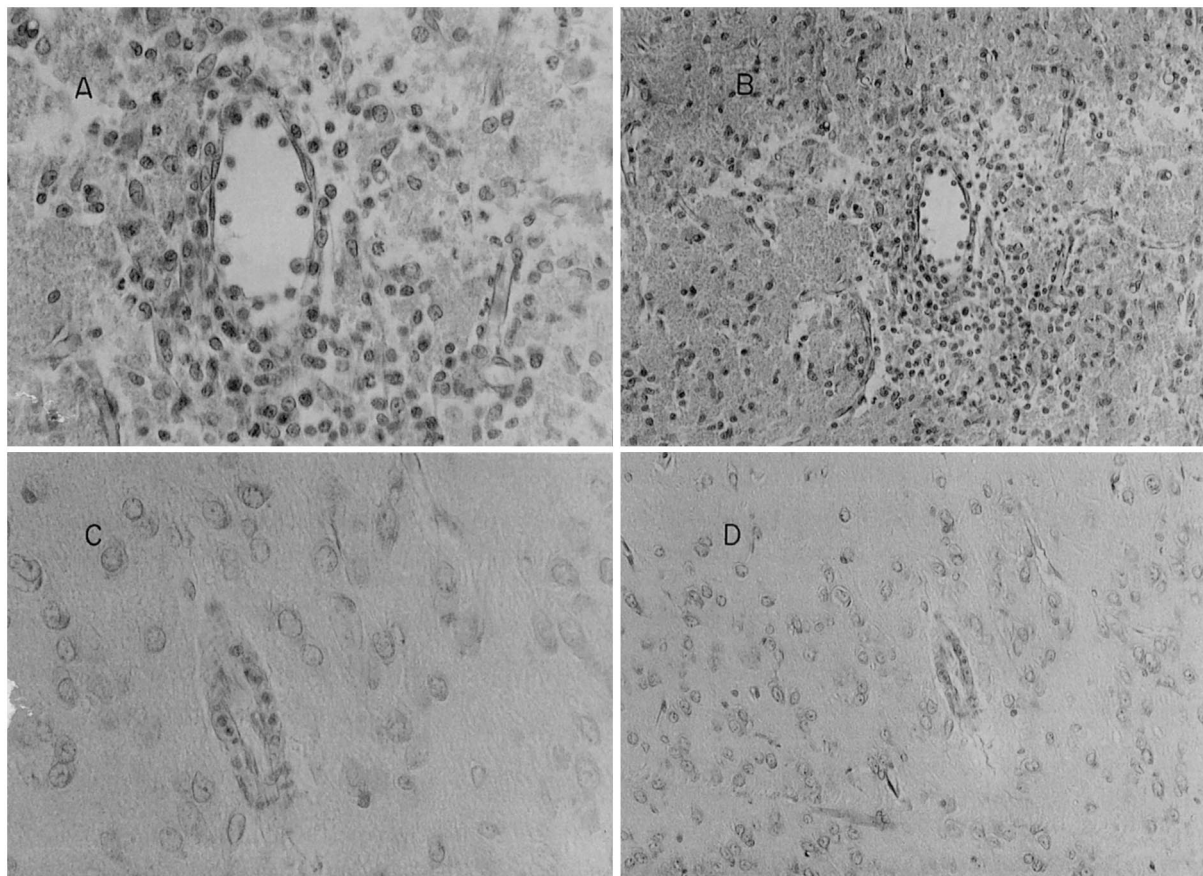


Fig. 1. (A) 400 X; (B) 200 X. Extensive neuronal damage and perivascular infiltration of lymphocytes within the piriform cortex of a rat that displayed overt seizures following lithium/pilocarpine injections and no overt symptoms of EAE. (C) 400 X; (D) 200 X. The piriform cortex of a rat that had been inoculated with spinal cord, had not received the lithium/pilocarpine, and, had exhibited no overt symptoms of EAE. Toluidine blue O stain.

neuronal dropout for the 106 structures for the left and right sides of the brain was not significant statistically. When all 382 structures (left and right side for each structure) that displayed some degree of neuronal loss were included, the correlation between numbers of foci and damage score for the neurons within these structures was weak ($\rho = 0.22$) but statistically significant ($P < 0.01$). In more than 90% of all cases, if perivascular infiltrations of lymphocytes were present, they were evident within the same structure on the left and right side of the brain. There was also no statistically significant association ($\rho = 0.11$) between the numbers of foci and the rating of the size (1–8 scale) of the focus.

The effects of gross disruptions of the blood–brain-barrier by intracerebral injections of fluid into the parenchyma were qualitative conspicuous for the six rats that received either the C 48/80 or isotonic saline. The rats that received this mast cell-degranulating compound showed more extensive infiltration of lymphocytes that effectively obscured the parenchymal structures within the entire hemisphere and was even evident by visual inspection of the slides without magnification. Although the concentration gradient for the dispersion of lymphocytes into parenchyma decreased as a function of distance from the injection site, they were still very evident at distances greater than 2 mm along the rostral–

caudal axis. At 1000 X (oil) magnification there was no discernable difference in morphology between these lymphocytes and those that were observed in the brains of the rats that had received injections of the low dosages of lithium/pilocarpine and spinal cord inoculations. There was no evidence of infiltrations in rats inoculated within the spinal cord emulsion but not injected with either of the dosages of lithium and pilocarpine. The microscopic appearance of the brains of these rats, employed as procedural controls, was not discriminably different from the brains of normal rats randomly selected from the colony.

4. Discussion

The results of this study clearly indicated that brains of rats in which overt seizures were induced experimentally (by a single systemic injection of 3.0 mE/kg of lithium followed 4 h later by 30 mg/kg of pilocarpine) after they were inoculated by a small amount of spinal cord (not sufficient to precipitate overt behavioral symptoms of EAE) showed multiple foci of lymphocytes around blood vessels. These foci of lymphocytes within brain parenchyma were particularly evident within structures involved with the limbic cortical–hippocampal system.

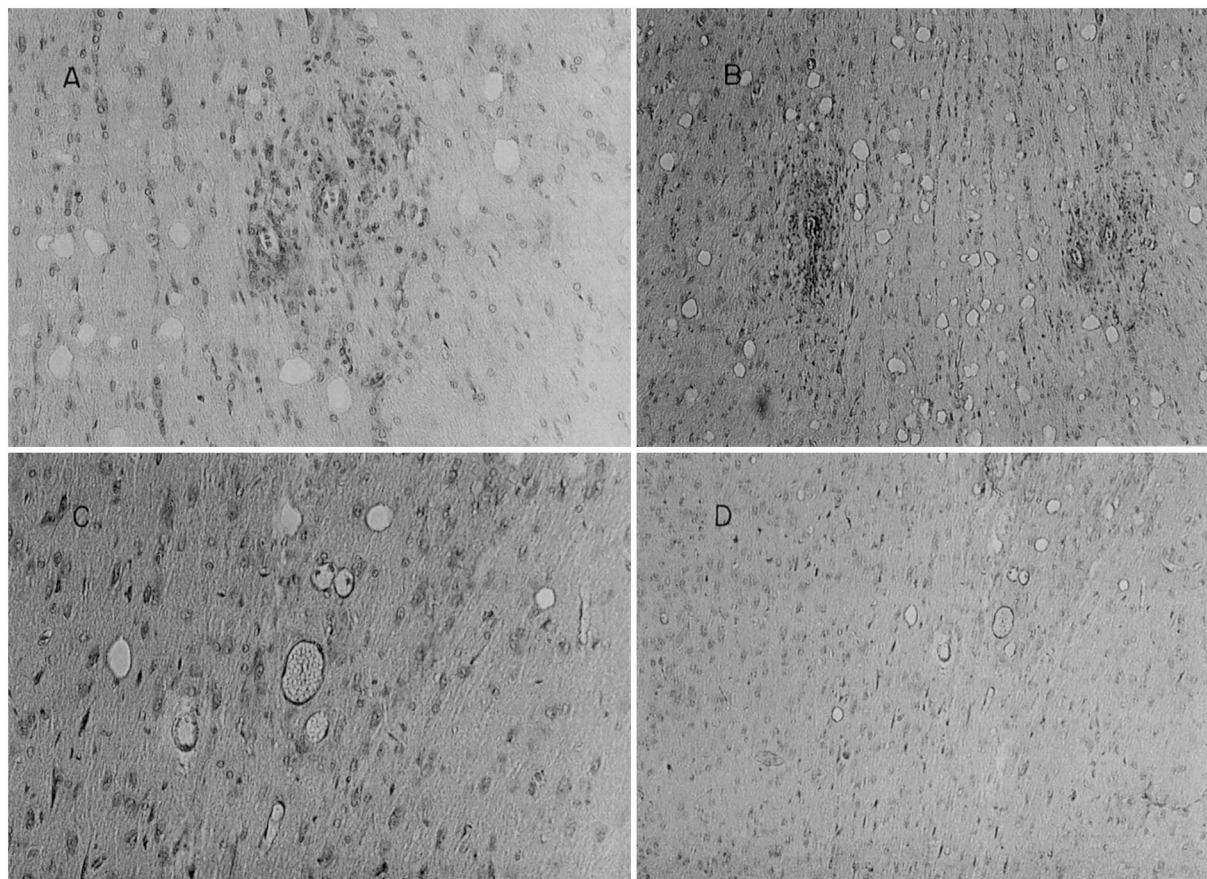


Fig. 2. (A) 200 X; (B) 100 X. Perivascular infiltration of lymphocytes into the white matter (fornix) from a rat that had been inoculated, had displayed no overt symptoms of EAE and had received only the lower dosages of lithium/pilocarpine (no overt seizures). (C) 200 X; (D) 100 X. The fornix of a rat that was inoculated with spinal cord only and showed no overt EAE symptoms (note integrity of blood vessels).

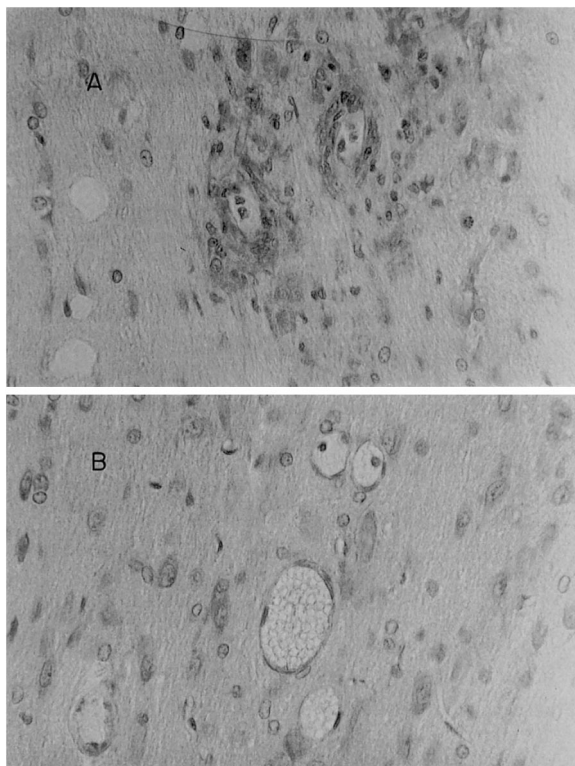


Fig. 3. (A) Enhanced magnification (400 X) of the fornix of a rat that had been inoculated, showed no overt EAE but had received the lowest (no overt seizures) dosages of lithium and pilocarpine. (B) The fornix of 2a procedural control rat.

The absence of a significant main effect for the severity of infiltration over time as a function of the latency of the seizure (0, 5 or 10 days) following the inoculation suggests that disruptions in the blood–brain-barrier any time during this period was associated with infiltrations of a similar magnitude. This suggestion is consistent with the observation that new multiple sclerotic lesions are preceded by disruptions of the blood–brain-barrier (BBB) which remained ‘open’ for about 4 weeks (Kwon and Prineas, 1994).

The observations that: (1) the width (numbers of layers) of infiltrations of lymphocytes into the parenchyma around foci and (2) the numbers of foci, were not significantly or very weakly correlated with the proportion of neuronal dropout associated with the overt seizures, indicate that the infiltrations were not due exclusively to non-

specific disruptions of the BBB. Structures that contribute to the limbic system were most affected. Area-specific responsiveness in the brain has been reported. For example Calza et al. (1997) have indicated that receptor-like immunoreactivity for nerve growth hormone (NGF) appears in perivascular areas and on neurons in only certain areas, such as within the cerebral cortices. They found a dramatic, differential regulation of NGF content and NGF receptor expression in different areas of the brain and spinal cord in EAE.

Although one would expect that transient metabolic disruptions sufficient to evoke overt seizures and neuronal loss would be sufficient to violate the BBB and allow infiltrations of cells, the occurrence of lymphocytes within the parenchyma of inoculated rats that received the low dosages of lithium and the cholinergic (muscarinic) agent pilocarpine suggests perhaps an unappreciated sensitivity of the BBB to what has been considered inconsequential stimuli. Small diffuse foci of lymphocytes were found within the limbic tract systems that were not evident even in brains which had sustained clear seizure-induced damage. These results are also consistent with our (Cook and Persinger, 1998) recent observations that these low dosages of lithium and pilocarpine interfere with long-term memory for spatial patterns but not the learning of spatial patterns.

We suggest that the observations of infiltrations of lymphocytes may be directly relevant to a persistent problem that occurs within a subpopulation of patients following mild closed head injury (Persinger and Makarec, 1993; Persinger, 1995) who often develop symptoms that have been described as indicators of an epileptic spectrum disorder (Roberts et al., 1992; Hines et al., 1995). Many of these patients develop chronic mild to moderate neuropsychological deficits even though their neurological examinations were normal. The symptoms include enhanced fatigue for skilled movements, disruptions in short-term memory, blurry vision (not resolvable by glasses), and poor concentration. Often they are attributed to ‘functional’ labels reserved for psychological or psychiatric disorders because the onset of the symptoms can occur weeks after the initial injury.

If our data are generalizable, then subclinical interactions between ingestion of elements from the environment (Persinger, 1987), pharmacological agents with muscarinic side-effects, and variations in the cellular immune system of these patients may result in more significant toxicological consequences than previously anticipated.

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